Biol Blood Marrow Transplant 21 (2015) 1555–1564

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

ASBMT American Society for Blood and Marrow Transplantation

Biology

Inhibition of the Immunoproteasome Subunit LMP7 with ONX 0914 Ameliorates Graft-versus-Host Disease in an MHC-Matched Minor Histocompatibility Antigen–Disparate Murine Model

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Article history:
Received 24 March 2015
Accepted 12 June 2015

Key Words:
Immunoproteasome
GVHD
Murine models
BMT

A B S T R A C T

In the current study we evaluated the effects of immunoproteasome inhibition using ONX 0914 (formerly PR-957) to ameliorate graft-versus-host disease (GVHD). ONX 0914, an LMP7-selective epoxyketone inhibitor of the immunoproteasome, has been shown to reduce cytokine production in activated monocytes and T cells and attenuate disease progression in mouse models of rheumatoid arthritis, colitis, systemic lupus erythematosus, and, more recently, encephalomyelitis. Inhibition of LMP7 with ONX 0914 in the B10.BR→CBA MHC-matched/minor histocompatibility antigen (miHA)-disparate murine blood and marrow transplant (BMT) model caused a modest but significant improvement in the survival of mice experiencing GVHD. Concomitant with these results, in vitro mixed lymphocyte cultures revealed that stimulator splenocytes, but not responder T cells, treated with ONX 0914 resulted in decreased IFN-γ production by allogeneic T cells in both MHC-disparate (B10.BR anti-B6) and miHA-mismatched (B10.BR anti-CBA) settings. In addition, a reduction in the expression of the MHC class I–restricted SIINFEKL peptide was observed in splenocytes from transgenic C57BL/6-Tg(CAG-OVA)916Jen/J mice exposed to ONX 0914. Taken together, these data support that LMP7 inhibition in the context of BMT modulates allogeneic responses by decreasing endogenous miHA presentation and that the consequential reduction in allogeneic stimulation and cytokine production reduces GVHD development.

INTRODUCTION

Immunotherapy in the form of allogeneic blood and marrow transplantation (allo-BMT) has proven to be one of the few curative treatments for patients suffering from a number of drug-resistant hematological malignancies. This therapy exploits the broad donor T cell repertoire targeting a diversity of defined and undefined allogeneic MHC antigens, non-MHC minor histocompatibility antigens (miHAs) derived from polymorphic intracellular proteins, and tumor-specific antigens presented by both MHC class I and class II molecules. Donor T cells play a central role in eliminating residual tumor cells that persist after conditioning regimens. However, they also facilitate patient immune reconstitution via homeostatic proliferation and provide first-line immunity against opportunistic infections while de novo bone marrow–derived immune cells slowly emerge after transplant. Although allo-BMT permits partial or complete remission in significant percentages of patients, it also comes with its limitations; in particular, acute graft-versus-host disease (GVHD) is a major cause of nonrelapse morbidity and mortality, affecting 40% to 60% of allo-BMT patients and accounting for 15% of deaths [1].

The proteasome is a large intracellular multicatalytic protease complex responsible for degradation of intracellular proteins and therefore critical for miHA presentation [2]. The 20S proteasome appears as a cylinder-like structure in various eukaryotes, consisting of 4 rings with 7 subunits each

Financial disclosure: See Acknowledgments on page 1563.

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http://dx.doi.org/10.1016/j.bbmt.2015.06.010
1083-8791/

[2,3]. Three of the β subunits, designated β1, β2, and β5, bear the active centers of the 20S proteasome. In hematopoietic cells and cells stimulated with IFN-γ and tumor necrosis factor-α (TNF-α), however, the catalytic subunits LMP2 (β1i), MECL-1 (β2i), and LMP7 (β5i) replace the constitutive subunits β1, β2, and β5 and are incorporated into the immunoproteasome [2,4]. The immunoproteasome regulates antigen presentation on MHC class I molecules, which displays peptides for surveillance of pathogen-infected cells by $CD8^+$ T cells and shapes the repertoire of that T cell subset in the thymus [4,5]. In addition to this role, the immunoproteasome also regulates cytokine production by immune cells [4,6]. More recently, a number of studies have shown that the immunoproteasome also plays a crucial role in T cell–mediated autoimmune diseases [3,6,7].

These results, together with preclinical GVHD studies demonstrating the beneficial effects of bortezomib [8-10], a proteasome inhibitor extensively used for the treatment of multiple myeloma [11,12], led to the current evaluation of the immunoproteasome inhibitor ONX 0914 (formerly PR-957) to ameliorate GVHD. ONX 0914, an LMP7-selective epoxyketone inhibitor of the immunoproteasome, has been shown to reduce cytokine production in activated monocytes and T cells and attenuate disease progression in mouse models of rheumatoid arthritis [7], colitis [6], systemic lupus erythematosus [13], and, more recently, encephalomyelitis [3]. Inhibition of LMP7 with ONX 0914 in the B10.BR→CBA MHC-matched/miHA-disparate murine BMT model caused a modest but significant improvement in the survival of mice experiencing GVHD. Concomitant with these results, in vitro mixed lymphocyte cultures (MLC) revealed that stimulator splenocytes, but not responder T cells, treated with ONX 0914 resulted in decreased IFN-γ production by allogeneic T cells in both MHC-disparate (B10.BR anti-B6) and miHA-mismatched (B10.BR anti-CBA) settings. In addition, a reduction in the expression of the MHC class I–restricted SIINFEKL peptide was observed in splenocytes from transgenic C57BL/6-Tg(CAG-OVA)916Jen/J mice exposed to ONX 0914. These data suggested that, in the context of GVHD, immunoproteasome inhibition via LMP7 likely modulates donor T cell allogeneic responses primarily via down-regulation of host antigen presentation of miHA, an effect that preferentially impacted cytotoxic $CD8^+$ T cell activity in our BMT model.

METHODS

Mice

Breeding pairs of B10.BR-H-wk H2-T18a/SgSnJJrep (B10.BR), C57Bl/6J (B6), and C57BL/6-Tg(CAG-OVA)916Jen/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our colony. Male CBA/J (CBA) mice were purchased from The Jackson Laboratory and used as recipients. All mice were housed in a sterile environment in microisolator cages and given autoclaved food and acidified water (pH 2.5) ad libitum. Both donor and recipient mice were used between 8 and 12 weeks of age. All protocols used in this study were approved by the Hackensack University Medical Center's Institutional Animal Care and Use Committee.

Flow Cytometry

Staining was performed using directly conjugated rat–anti-mouse mAbs (BD Pharmingen, San Jose, CA) diluted to 1:100 in FACS buffer (1% BSA with 0.02% sodium azide in 1× PBS) directed to CD4 PE (clone RM4-5), CD8 PC5 (clone 53-6.7), and B220 FITC (clone RA3-6B2). Anti-H2Kb-SIINFEKL mAb (clone eBio25-D1.16) was purchased from eBioscience (San Diego, CA). The FC500 flow cytometer (Beckman Coulter, Miami, FL) was used for all acquisitions and analyses.

Preparation of Cells for GVHD Experiments

Donor lymphocytes were prepared from pooled RBC-lysed spleens and lymph nodes enriched for T cells by a 37°C incubation with anti-CD24 mAb (J11d.2; 1:500) and guinea pig complement (C′) to deplete B cells. $CD8^+$ T cells were then depleted from the T cell–enriched population using $CD8^+$ specific mAb (3.168; 1:100) to augment the $CD4^+$ population. Anti-Thy1 mAb + C′ treated bone marrow (ATBM) cells were produced from donor mice by flushing the bone marrow cells from the femurs, followed by incubation with anti-Thy1 mAb (J1j; 1:100) and C′.

GVHD Experiments

CBA recipient mice were exposed to lethal irradiation (11 Gy, split dose, 4 hours apart) using a ^{137}Ce source (Gammacell 40 Exactor; NDS Nordion, Canada). Mice were then transplanted with 2×10^6 B10.BR ATBM cells alone or in combination with 1×10^7 enriched B10.BR T cells. ONX 0914 was administered i.v. for the day 0 to 2 treatment in our first experiment and s.c. for all subsequent treatments and experiments. Responses were comparable by either administrative route, but multiday s.c. injections were technically easier to perform. Mice were examined daily for morbidity and mortality and weighed twice weekly.

ELISpot Assay

Millipore (Billerica, MA) 96-well multiscreen plates with Immobilon-P membrane were coated with affinity-purified anti-mouse IFN-γ mAb (AN-18; 10 μg/mL; eBioscience) at 4°C overnight. Responder cell populations were prepared by harvesting peripheral lymph nodes and enriching for T cells by B cell depletion and, when necessary, $CD8^+$ enrichment by CD4 depletion. RBC-lysed splenocytes were irradiated (15 Gy) and used as stimulators. Compound treated groups were incubated for 1 hour at 37°C, 5% CO_2, with either .1% DMSO vehicle, 300 nM ONX 0914 (a LMP7-selective concentration [7]) solubilized in .1% DMSO, or 125 nM PR-825 (a β5 subunit selective concentration [6]) solubilized in .1% DMSO, followed by 3 washes with cRPMI before plating.

Coated ELISpot plates (Millipore, Billerica, MA) were washed and blocked for 1 hour at 37°C, 5% CO_2 with cRPMI before plating the cells in an R:S ratio of 1:2 in cRPMI (RPMI 1640 media [Mediatech, Herndon, VA] containing 10% FBS [Hyclone, Logan, UT], 2 mM L-glutamine, penicillin [50 IU/mL[/streptomycin [50 μg; Mediatech] and 2-ME) for 48 hours at 37°C, 5% CO_2. After incubation, the plates were washed with 1× PBS containing .01% Tween-20 (Sigma-Aldrich, St. Louis, MO).

Biotinylated anti-mouse IFN-γ mAb (clone R4-6A2; 1 μg/mlL; eBioscience) was incubated for 2 hours in the dark at room temperature, followed by Tween-20 washes as described above. The plate was then incubated with a 1:1000 dilution of Avidin-HRP (eBioscience) in 1× PBS for 1 hour at room temperature. 3,3′-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich) substrate solution was used to develop the plate for 10 minutes at room temperature, followed by washing and spot enumeration using an Immunospot plate reader, software version 3.2 (Cellular Technology Limited, CTL, Shaker Heights, OH). Spot counts from a minimum of 4 wells per experiment were averaged and statistical analysis performed. Mouse strains and T cell populations are indicated for each experiment in the appropriate figure legend, as cited below.

Antigen Presentation Assay

Spleens from C57BL/6-Tg(CAG-OVA)916Jen/J mice were harvested and RBCs lysed. Splenocytes were either left untreated or treated with .1% DMSO or 300 nM ONX in .1% DMSO for 1 hour at 37°C, 5% CO_2. After treatment, cells were washed 3 times and plated with cRPMI at 37°C, 5% CO_2. SIINFEKL expression was analyzed by flow cytometry at 24 and 48 hours post-treatment.

Histopathology

At days 5 and 12 post-transplantation, GVHD target organs (liver, spleen, small and large intestine) were harvested into 4% paraformaldehyde, which was washed and replaced with 70% ethanol after 24 hours. Samples were sent to the Digital Imaging and Histology Core at Rutgers-NJMS Cancer Center (Newark, NJ) for paraffin embedding, sectioning, slide preparation, and H & E staining. GVHD scoring was performed by a veterinary pathologist. A semiquantitative scaling from 0 to 5 was implemented based on histopathological findings, as follows: normal = 0, minimal = 1, mild = 2, moderate = 3, moderately severe/marked = 4, and severe/high = 5. A half score was considered for the findings that fell between severities described earlier. Cumulative histopathology scores were calculated based on the sum of individual changes of various parameters in each organ (villous blunting, crypt cell hyperplasia, crypt cell apoptosis, and mucosal and submucosal inflammation in the small intestine; Goblet cell depletion, crypt cell hyperplasia, crypt cell apoptosis, and mucosal and submucosal inflammation in large intestine; and bile duct degeneration and inflammation in liver). Each experiment consisted of 3 to 4 mice per group. Images were visualized using an Olympus BX61 microscope (Waltham, MA) and images captured with CellSense Standard software (Olympus, Waltham, MA).

Proliferation Analyses

A pilot experiment was conducted to evaluate and compare the proliferative capacity of donor T cells in recipient mice treated with either ONX 0914 or bortezomib (Supplemental Figure 1). To this end, B10.BR cells isolated from peripheral lymph nodes were labeled with 5 μM of the cell proliferation dye eFluor 670 (eBioscience) according to the manufacturer's instructions. B6 recipient mice (3 to 4 mice per group) were exposed to lethal irradiation (11 Gy, split dose, 4 hours apart) and inoculated with 2×10^7 eFluor670-labeled B10.BR T cells and treated on days 0, 1, and 2 with either ONX 0914, bortezomib, or vehicle (Captisol; Ligand Technology, La Jolla, CA). On day 4 mice were killed, and splenic cells were collected for flow cytometric analyses.

Statistical Analyses

For multiple comparisons (>2 groups), 1-way repeated-measures analysis of variance (ANOVA) was conducted, followed by Tukey's multiple comparison test on individual pairs to determine significant differences among groups when ANOVA test was statistically significant. Student *t*-test was used when comparing 2 groups. A $P < .05$ was considered significant. Prism software (GraphPad, La Jolla, CA) was used to perform analyses.

RESULTS

LMP7-Selective Epoxyketone Inhibition Using the Immunoproteasome Inhibitor ONX 0914 Prolongs the Survival of Transplanted Mice in the B10.BR into CBA miHA-Disparate BMT Model

The potential use of ONX 0914 as an immunotherapeutic agent to ameliorate GVHD was evaluated in the MHC-matched/miHA-disparate $CD8^+$ T cell–mediated B10BR→CBA murine BMT model. ONX 0914 was administered at a concentration of 8 mg/kg (a dose that preferentially targets LMP7 and to a lesser degree LMP2) on days 0, 1, and 2 post-BMT either in full dose (once a day), split dose (twice a day, 6 hours apart), or split dose on days 0, 1, 2, 3, and 4. ONX 0914 (days 0, 1, and 2, full dose) resulted in a modest but statistically significant prolongation of the survival of treated mice compared with that of the nontreated (GVHD) recipients (Figure 1A; ONX 0914 median survival time (MST) = 24 days versus GVHD MST = 18.5 days; $P = .023$). No other regimen yielded statistically significant improvement in the MST of GVHD mice. Weight loss was monitored in survival experiments as an indicator of GVHD severity (Figure 1B). No statistical differences were found between GVHD and ONX 0914 groups or between ATBM and ATBM + ONX 0914 recipient mice.

ONX 0914 Preferentially Affects Stimulator Cells and Modulates the Production of INF-γ by Responder T Cells in MLCs of MHC-Mismatched and miHA-Disparate BMT Models

To better understand the mechanisms by which immunoproteasome inhibition improved the survival of transplanted mice undergoing GVHD, we examined the effect of ONX 0914 treatment in MLC from the B10.BR anti-B6 MHC-mismatched and the B10.BR anti-CBA miHA-disparate BMT models. In brief, for the B10.BR anti-B6 model, B6 stimulator (S) splenocyte populations were either exposed to irradiation (15 Gy) and treated with vehicle (DMSO, .1%) or ONX 0914 (300 nM) or treated before irradiation, as indicated (Figure 2A and B). We chose to evaluate radiation timing (ie, before or after treatment) to better recapitulate the conducted in vivo experiments where mice received treatment post-irradiation and to determine if treatment pre-irradiation could have been beneficial to their survival. After treatment, cells were washed 3 times with PBS and plated along with B10.BR responder (R) T cells as described, at an R:S ratio of 1:2 (2×10^5 to 4×10^5). For some experimental conditions, responder T cells were also treated with ONX 0914 (300 nM) for 1 hour and washed 3 times with PBS before plating (Figure 2A and B).

The results from these experiments indicate the following. First, treatment of B6 stimulator cells with ONX 0914 significantly decreased the production of IFN-γ by alloreactive B10.BR responder T cells, irrespective of irradiation order ($P < .05$ before irradiation and $P < .05$ after irradiation). Second, ONX 0914 treatment of only B10.BR responder T cells had no effect on their IFN-γ production

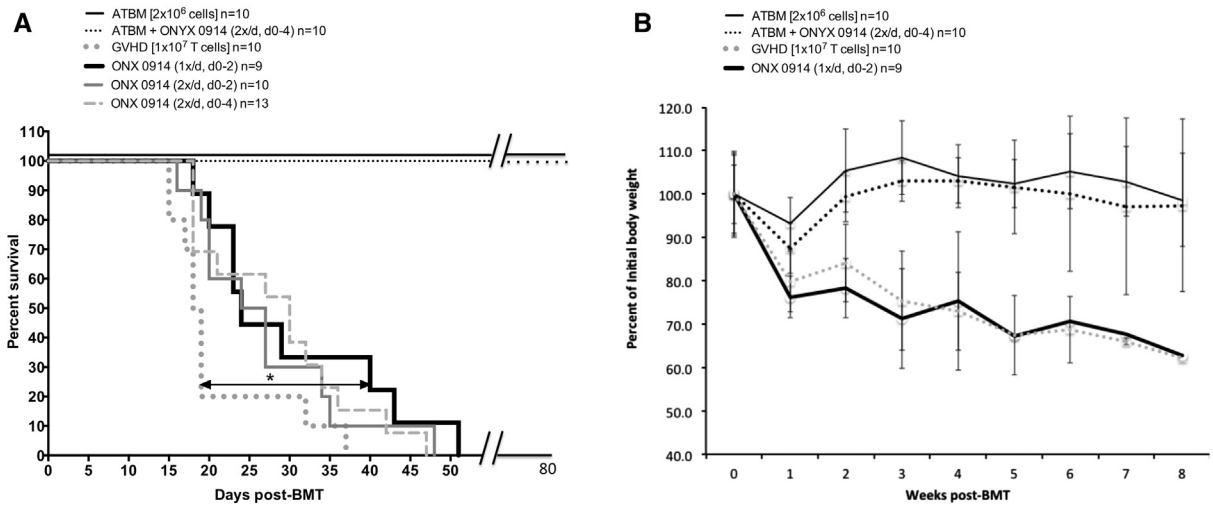


Figure 1. Effect of ONX 0914 on the survival rate of transplanted mice in the B10.BR into CBA miHA-disparate BMT model. (A) CBA recipient mice were exposed to lethal irradiation (11 Gy, split dose) and transplanted with 2×10^6 B10.BR anti-Thy1 mAb + C′ treated bone marrow (ATBM) cells in combination with 1×10^7 enriched B cell–depleted B10.BR T cells (GVHD control and ONX 0914 treated groups). ONX 0914 was dissolved in Captisol and administered to mice as an s.c. or i.v. bolus (full dose, 1×/d, or split dose, 2×/d, 6 hours apart at 8 mg/kg) on days 0, 1, and 2 (d0-2) or days 0, 1, 2, 3, and 4 (d0-4) (split dose, 2×/d, 6 hours apart) post-BMT. Shown are 2 pooled experiments with 9 to 3 mice per group. Statistical significance between survival curves was determined using the log-rank (Mantel-Cox) test. GVHD versus ONX 0914 (full dose, d0-2), $^*P = .023$. Additionally, ATBM versus GVHD or any of the ONX 0914–treated groups, $P < .01$, ATBM + ONX 0914 versus GVHD or any of the ONX 0914 treated groups, $P < .01$. (B) Percent of initial body weight of control ATBM, ATBM + ONX 0914 (split dose, d0-4), and GVHD groups compared with that of best ONX 0914 regimen (full dose, d0-2). The mean ± standard deviation percent initial body weight of surviving mice in each group was derived relative to the mean weight of the group on d0.

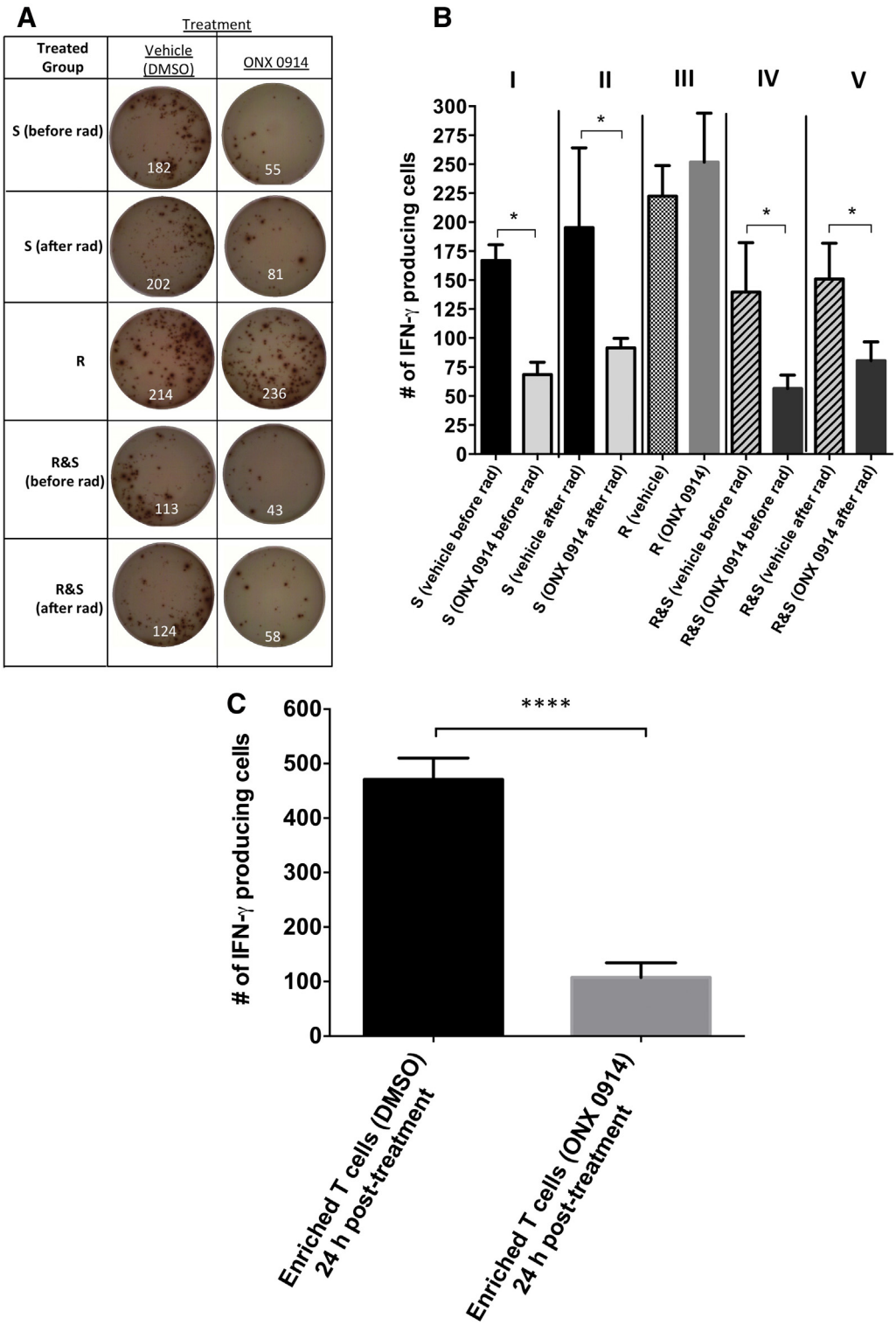


Figure 2. Effect of ONX 0914 on the production of IFN-γ by allogeneic T cells in MHC-mismatched B10.BR anti-B6 MHC MLCs. Lymph node cells isolated from B10.BR mice were T cell enriched by B cell depletion and used as responders (R) in MLCs. Splenocytes from B6 mice were prepared as described and exposed to irradiation (15 Gy) before use as stimulators (S). MLCs were plated at a, R:S ratio of 1:2 (2×10^5 to 4×10^5) and incubated for 48 hours before measuring INF-γ production by responder cells using ELISpot assays. ONX 0914 (300 nM) was solubilized in a DMSO vehicle (.1%) and stimulators, responders, or both responders and stimulators were treated for 1 hour at 37°C either before or after irradiation (rad) and compared with vehicle (DMSO) treatment. All cells were washed 3 times with cRPMI-1640 media before plating. (A) Representative wells showing spot counts of IFN-γ–producing cells. (B) IFN-γ spot counts under different culture conditions as specified on the *x*-axis. Experiments were repeated 3 times with 4 to 8 wells per experiment, and data are from a representative experiment. Statistical analyses were performed using a nonparametric Mann-Whitney U test to compare spot counts between DMSO and ONX 0914 treatments under the specified conditions. $^*P < .05$. The percent decrease in spot counts between DMSO and ONX 0914 treatments was as follows: I = 59%, II = 53%, III = N/A, III = 59%, and IV = 45%. (C) IFN-γ spot counts after a 48-hour MLC (R:S ratio of 1:2 [3×10^5 to 6×10^5]) where B6 stimulators were pretreated for 24 hours (at 37°C and 5% CO_2) with either ONX 0914 (300 nM) or vehicle (DMSO, .1%) before plating. Data are results of 1 experiment with 12 wells. The Mann-Whitney U test was used to compare spot counts between DMSO and ONX 0914 treatments. $^{****}P < .0001$. The percent decrease in spot counts between DMSO and ONX 0914 treatments was 77%.

(222.5 ± 26.34 counts in vehicle-treated versus 251.8 ± 42.37 counts in ONX 0914–treated cells). Third, treatment of both responder and stimulator cells, before or after irradiation, resulted in a significant count reduction of approximately 60% ($P < .05$), which was equivalent to that of treatment of stimulators alone.

Next, we tested the effects of incubating B6 splenocyte cells for a period of 24 hours after the 1-hour exposure to either vehicle or ONX 0914. Although a significant reduction in the number of spot counts was also observed as a result of this culture approach (Figure 2C, $P < .0001$), the 24-hour interval did not completely abrogate the production of IFN-γ by B10.BR allogeneic T cells (ONX 0914 presented with 107 spot counts versus 470 spots with DMSO-treated splenocytes; a 77% decrease).

Because other proteasome inhibitors such as bortezomib can mediate equivalent inhibition of the β5 and LMP7 subunits, we investigated the specificity of the observed response to the inhibition of the LMP7 subunit by comparing the effects of the selective β5 inhibitor PR-825 with that of ONX 0914 (Figures 3 and 4) on the production of IFN-γ by allogeneic T cells. The results of these assays indicated that PR-825 had no effect on either the MHC-mismatched (Figure 3) or miHA-disparate (Figure 4) BMT

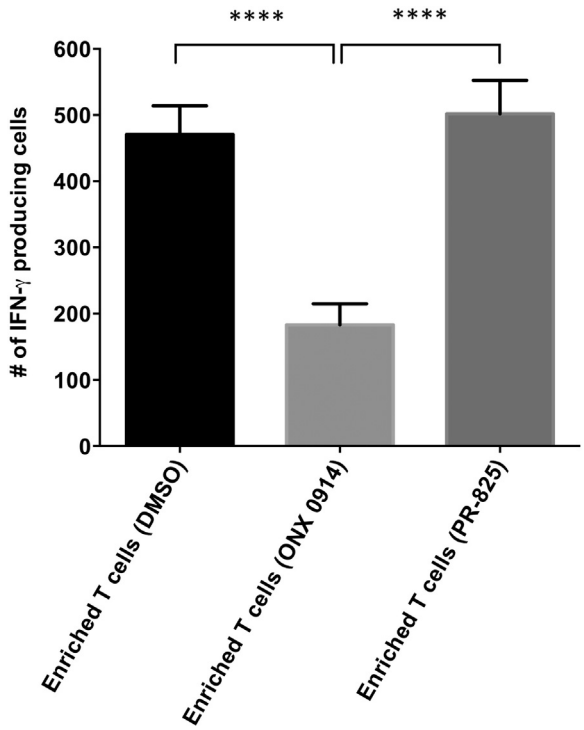


Figure 3. Comparison of ONX 0914 and PR-825 effects on the production of IFN-γ by allogeneic T cells in MHC-mismatched B10.BR anti-B6 MHC MLCs. Lymph node cells isolated from B10.BR mice were T cell enriched by B cell depletion and used as responders (R) in MLCs. Splenocytes from B6 mice were prepared as described and exposed to irradiation (15 Gy) before use as stimulators (S). MLCs were plated in an R:S ratio of 1:2 (3×10^5 to 6×10^5 cells) and incubated 48 hours at 37°C before measuring INF-γ production by responder cells using ELISpot assays. B6 stimulators were incubated for 1 hour at 37°C in vehicle (DMSO, .1%), ONX 0914 (300 nM), or PR-825 (125 nM) and washed 3 times with cRPMI-1640 before plating. Experiments were repeated 3 times with 4 to 8 wells per experiment, and data are from a representative experiment. Statistical analysis was performed using 1-way ANOVA to compare IFN-γ spot counts between DMSO, ONX 0914, and PR-825 treatments, followed by Tukey's multiple comparison test on individual pairs when ANOVA test was statistically significant ($P < .05$). ****$P < .0001$.

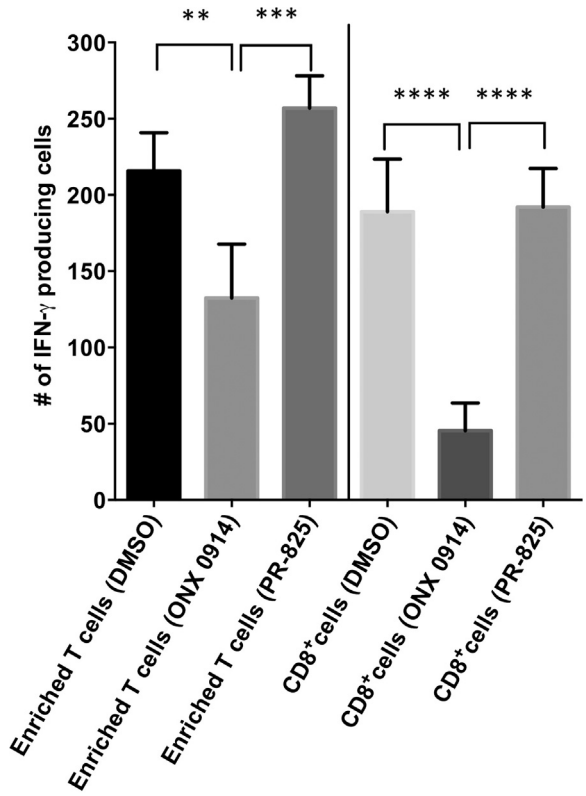


Figure 4. Comparison of ONX 0914 and PR-825 effects on the production of IFN-γ by allogeneic T cells in miHA-mismatched BB10.BR anti-CBA MLCs. Enriched T cells (B cell depleted) or $CD8^+$ T cells (B cell and CD4 depleted) from B10.BR mice were used as responders (R) in MLCs. Splenocytes from CBA mice were prepared as described and exposed to irradiation (15 Gy) before use as stimulators (S). MLCs were plated in an R:S ratio of 1:2 (3×10^5 to 6×10^5 cells) and incubated 48 hours at 37°C before measuring INF-γ production by responder cells using ELISpot assays. CBA stimulators were incubated for 1 hour at 37°C in vehicle (DMSO, .1%), ONX 0914 (300 nM), or PR-825 (125 nM) and washed 3 times with cRPMI-1640 before plating. Experiments were repeated 3 times with 4 to 8 wells per experiment, and data are from a representative experiment. Statistical analysis was performed using 1-way ANOVA to compare IFN-γ spot counts between DMSO, ONX 0914, and PR-825 treatments, within the enriched T cell or $CD8^+$ T cell group, followed by Tukey's multiple comparison test on individual pairs when ANOVA test was statistically significant ($P < .05$). **$P < .01$, ***$P < .001$, ****$P < .0001$.

tested models. Furthermore, because B10.BR→CBA GVHD is dominantly mediated by $CD8^+$ T cells [14], we also conducted MLC and ELISpot assays to determine IFN-γ production by enriched $CD8^+$ B10.BR T cells against CBA stimulators treated with either DMSO, ONX 0914, or PR-825 (Figure 4). Once more, ONX 0914 significantly reduced the spot counts (*P* values in figure legends), whereas PR-825 had no effect.

ONX 0914 Reduces the Production of IFN-γ by CD3/CD28 Polyclonally Stimulated T Cells

To determine the effect of ONX 0914 on T cell activation, we polyclonally stimulated ONX 0914–treated B10.BR T cells with antibodies directed to CD3 and CD28. Although we found that direct treatment of T cells with ONX 0914 had no impact on the production of IFN-γ upon allo-stimulation, cytokine production of polyclonally stimulated T cells was significantly decreased by selective LMP7 inhibition with ONX 0914 compared with vehicle treatment by approximately 50% ($P = .038$, Figure 5).

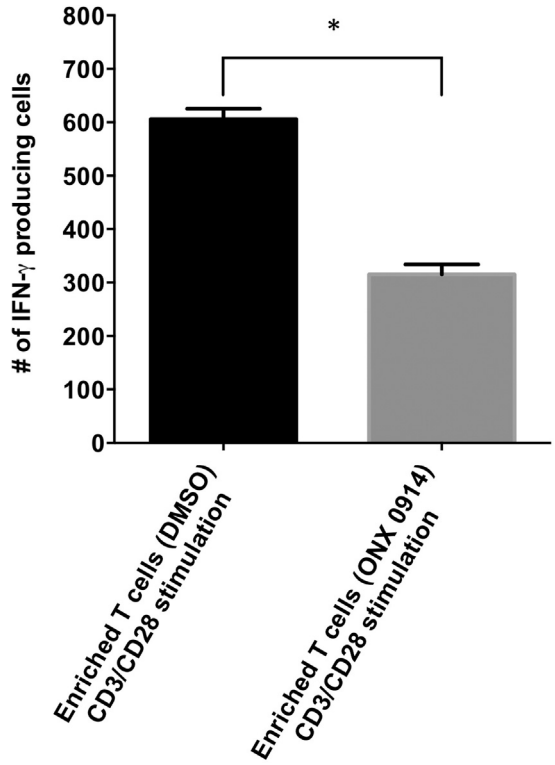


Figure 5. Effect of ONX 0914 on the production of IFN-γ by CD3/CD28 stimulated T cells. B10BR lymph nodes were B cell depleted and 2×10^5 cells were stimulated for 24 hours (at 37°C and 5% CO_2) with 5 μg/mL soluble purified CD3 mAb and 5 μg/mL purified CD28 mAb, followed by ELISpot assay development. Experiments were repeated twice with 4 to 8 wells per experiment, and data are from a representative experiment. The Mann-Whitney U test was used to compare spot counts between DMSO and ONX 0914 treatments. $^*P < .05$.

CBA Recipient Mice Treated with ONX 0914 Exhibit Comparable GVHD Pathological Scoring with Control Nontreated Mice

A separate set of BMT recipient mice were killed on days 5 and 12 post-BMT for pathological evaluation to determine if the improved survival of ONX0 914–exposed groups (Figure 1A) was associated with a concomitant histological evidence of GVHD amelioration. The percent of initial body weight (Figure 1B) was found to be equivalent between groups (ie, ATBM versus ATBM + ONX0914 and GVHD versus ONX 0914 8 mg/kg [full dose, days 0, 1, and 2]), yet there was a slight tendency for increased weight loss in groups exposed to ONX 0914. Furthermore, quantitative histopathological assessment of GVHD also indicated no difference between treated and nontreated groups at either time point. Small intestine, large intestine, and liver were analyzed and scored by a trained pathologist based on villous blunting, crypt cell hyperplasia, crypt cell apoptosis, and inflammation (small intestine); crypt hyperplasia, crypt cell apoptosis, inflammation, and Goblet cell depletion (colon); and bile duct degeneration and inflammation (liver).

Scoring results and statistical comparisons between groups can be found in Figure 6A. Figure 6B is a representative H & E histological image (day 12) of the small intestine, colon, and liver for each group. As expected, GVHD scoring was higher in mice challenged with T cells (GVHD and ONX 0914 groups) compared with mice receiving only ATBM cells (ATBM and ATBM + ONX 0914 groups), but only on day 12 were the means significantly different (ie, ATBM versus GVHD, and ATBM + ONX 0914 versus ONX 01914, $P < .001$). Noticeably, on day 5, the mean scoring for the ONX 0914 group was lower, albeit not significantly, compared with that of GVHD mice (15.5 versus 14.25, respectively). This difference, however, was found to have reverted by day 12 (ie, ONX 0914 mean score = 23.13 versus GVHD group = 21). Of interest, 1 mouse in the ATBM + ONX 0914 group presented with increased pathological scoring on day 12 as a result of extensive inflammation and Goblet cell depletion (Figure 6A, circled data point).

ONX 0914 Inhibits MHC Class I–Restricted Antigen Presentation

It was reported that presentation of the endogenously expressed LMP7-dependent male antigen UTY 246–254 epitope could be blocked by exposure to ONX 091475 [7]. Furthermore, in immunoproteasome triple knockout mice, MHC class I–restricted SIINFEKL peptide expression was significantly reduced compared with wild-type cells [15]. SIINFEKL peptide, the immunodominant epitope from ovalbumin in the context of H-$2K^b$, is constitutively expressed on transgenic C57BL/6-Tg(CAG-OVA)916Jen/J (B6-SIINFEKL) mice and can elicit $CD8^+$ T cell–specific responses. Therefore, we also tested the effect of ONX 0914 on SIINFEKL presentation. To this end, RBC-lysed splenocytes from B6-SIINFEKL mice were treated for 1 hour at 37°C, as described, and washed and incubated (37°C and 5% CO_2) for 3, 24, and 48 hours post-treatment before flow cytometric analysis to determine the percentage decrease in MHC-bound SIINFEKL expression. As early as 3 hours post-treatment, SIINFEKL expression was reduced by approximately 16% with respect to DMSO exposure and peaked at 24 hours with an approximate 27% reduction (Figure 7, $P = .039$).

DISCUSSION

Proteasome inhibition has emerged as a useful anticancer treatment for a number of hematological malignancies, including multiple myeloma; solid tumors, such as head and neck; and colorectal, prostate, and non–small cell lung cancer [16]. Although proteasome inhibitors represent a novel class of anticancer therapy with preferential induction of apoptosis in transformed cells [17,18], their therapeutic potential has also been extended to T cell–mediated diseases because of their effects on cytokine production and proliferation of lymphocytes as well as antigen presentation. Bortezomib in particular, which targets the constitutive proteasome and the immunoproteasome, has been shown to prevent GVHD while preserving graft-versus-leukemia (GVL) responses in tumor-bearing mice after allogeneic BMT [8,9]. More recently, a number of novel proteasome inhibitors preferentially targeting subunits of the immunoproteasome have been developed to improve the pharmacological profile of these drugs while decreasing unwanted toxicity [5,7,19,20]. In particular, the LMP7-selective epoxyketone proteasome inhibitor ONX 0914 has been shown efficacious in various experimental models of autoimmune disease, including arthritis [7], colitis [6], and experimental autoimmune encephalomyelitis [3].

Based on these findings, we tested the effects of immunoproteasome suppression by ONX 0914 in the development of GVHD using the B10.BR→CBA MHC-matched, miHA-disparate BMT model. In this model, LMP7-selective inhibition with ONX 0914 resulted in a modest but significant improvement in the survival rate of treated mice

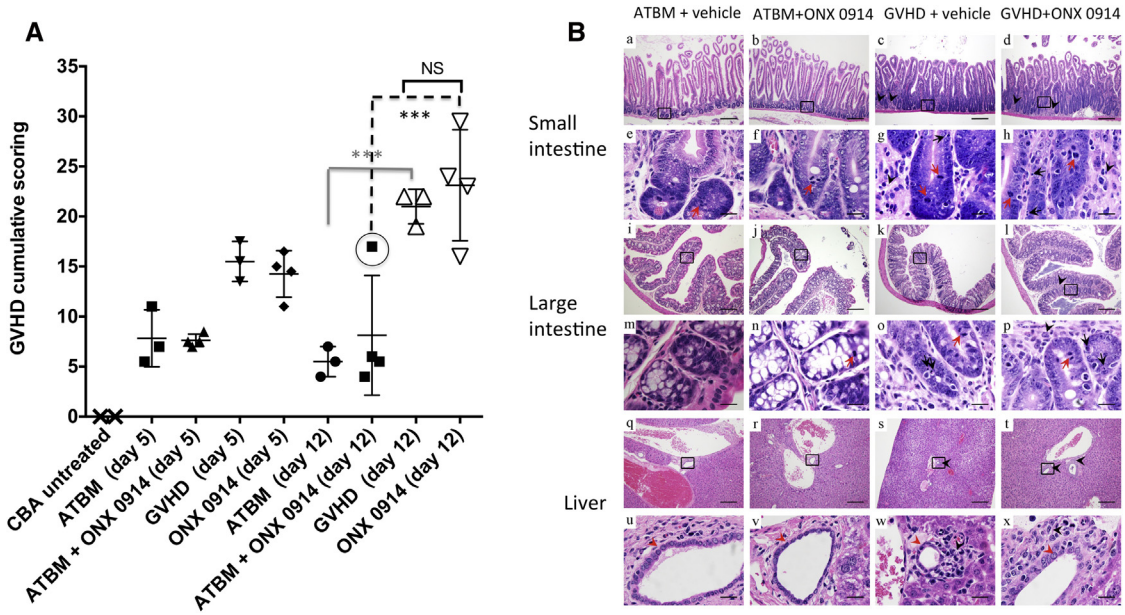


Figure 6. Histological evidence of GVHD in CBA recipients treated with ONX 0914. CBA mice were exposed to lethal irradiation (11 Gy, split dose) and transplanted with 2×10^6 ATBM cells alone or in combination with 1×10^7 $CD8^+$ T cells from B10.BR donor mice. ONX 0914 was administered (full dose, 8 mg/kg) on days 0, 1, and 2 post-BMT as previously described, and mice were killed on days 5 and 12 post-BMT for pathological analyses of the small intestine, colon, and liver. (A) Quantitative histopathological assessment of GVHD. Small intestine, colon, and liver were analyzed and scored based on villous blunting, crypt cell hyperplasia, crypt cell apoptosis, and inflammation (small intestine); crypt hyperplasia, crypt cell apoptosis, inflammation, and Goblet cell depletion (colon); and bile duct degeneration and inflammation (liver). Data points represent the mean scores of 3 to 4 mice ± standard deviation per group. Statistical analysis was performed using 1-way ANOVA to compare the mean of the cumulative GVHD scoring between groups, followed by Tukey's multiple comparison test on individual pairs when ANOVA test was statistically significant ($P < .05$), ***$P < .001$, NS indicates not significant. Circled data point highlights an animal in the ATBM + ONX 0914 group on day 12 presenting with increased pathological scoring. (B) Histological staining (H & E) of small intestine, large intestine, and liver on day 12 day post-BMT. Representative images from small intestine (a-h), large intestine (i-p), and liver (q-x) of ATBM (a, b, e, f, i, j, m, n, q, r, u, v) and GVHD (c, d, g, h, k, l, o, p, s, t, w, x) mice either treated with vehicle alone (a, c, e, g, i, k, m, o, q, s, u, w) or with ONX 0914 (b, d, f, h, j, l, n, p, r, t, v, x). Significant villous blunting, crypt cell hyperplasia, and apoptosis are evident in GVHD mice. Minimal to mild degree of infiltration of inflammatory cells were found in hepatic portal triads, but the epithelial integrity of the ducts were intact (*red arrowheads*). *Black arrowheads* indicates the inflammatory foci. *Black arrows* and *red arrows* indicate representative apoptotic cells and mitotic figures, respectively. Lower panels are 10× magnification of upper panels. Scale bar for upper and lower panels 200 μM and 20 μM, respectively.

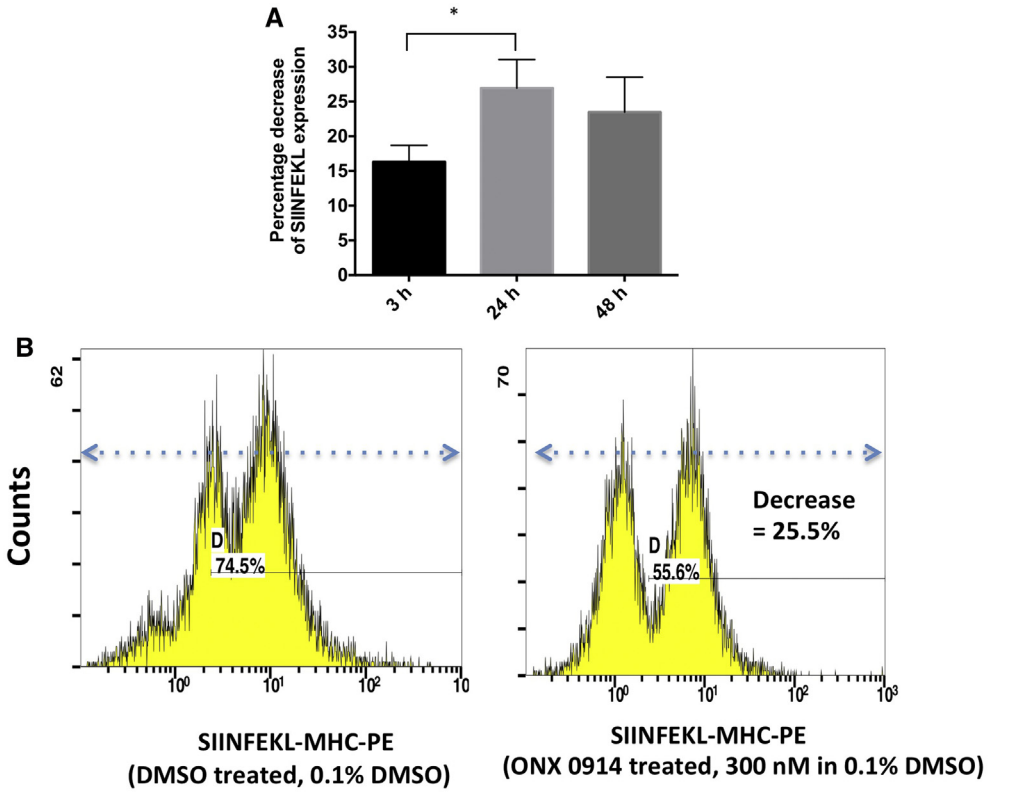
(Figure 1A). This beneficial effect, however, did not correlate with decreased weight loss (Figure 1B) or amelioration of pathological signs at either day 5 or 12 post-BMT (Figure 6A), for all mice undergoing GVHD, irrespective of treatment, manifested with comparable levels of GVHD cumulative scoring (Figure 6A). That notwithstanding, on day 5, mice treated with ONX 0914 exhibited a modest decrease in pathological presentation, although not statistically significant (Figure 6A), suggesting the impact of treatment might have taken place during or immediately after drug exposure but that upon withdrawal the beneficial effects of immunoproteasome suppression was lost.

Of interest, prolonged daily regimens on days 0 to 4; days 0, 2, 4, 6, 8, and 10 (data not shown); or split-dose daily treatment to try to maintain constant levels of drug presence failed to improve the survival of CBA recipient mice. In addition, we also observed that mice in the ATBM control group treated daily with ONX 0914 (days 0 to 4) presented with greater weight loss at earlier time points, a trend that continued up until the termination of the experiment (Figure 1B), and that at least 1 mouse in the ATBM + ONX 0914 group sampled for pathology had greater GVHD cumulative scoring on day 12 (Figure 6A). Taken together, these data support the notion of an early benefit upon ONX 0914 administration in GVHD (<day 5) that was abrogated by day 12 and that extended treatment with this LMP7 inhibitor may indeed be counterproductive.

Others have also reported [10,21] that the ability of bortezomib to prevent GVHD was subjected to its administration immediately after BMT (days 0 to 3) and that later treatment of bortezomib, during GVHD development, or prolonged administration of this inhibitor resulted in a GVHD-like lethal toxicity resulting in marked acceleration of the disease. Of interest, this toxicity was correlated with an increased presence of inflammatory cytokines (such as IL-1β, IL-6, and TNFα), the expression of TNF-receptor 1 [10], and allogeneic $CD4^+$ T cells were found to be the key cell population responsible for these side effects [8]. A more selective inhibitor of the NF-κB pathway (PS-1145), however, did induce toxicity in a murine model of GVHD when used either at later time points post-BMT or in a prolonged regimen [21].

Furthermore, in our experimental model, a higher but less selective dosage of ONX 0914 (10 mg/kg) also proved detrimental and resulted in a shorter MST (data not shown). Although we did not conduct additional experiments to elucidate the lack of GVHD improvement upon prolonged or increased treatment, our combined results suggested that selective targeting of the immunoproteasome provided no outstanding advantage compared with bortezomib, which when administered early on after transplant in the BALB/c→B6 MHC-mismatch model [9] resulted in a significant increase in the MST of recipient mice or complete abrogation of GVHD, depending on the number of transplanted donor T cells.

Next, we investigated other potential mechanisms by which ONX 0914 could have halted GVHD progression. In this regard, we tested its effects on the production of IFN-γ by allogeneic T cells in B10.BR anti-CBA as well as B10.BR anti-B6



C

Region	MFI
ALL	9.64
D	12.5

Region	MFI	% Decrease (MFI)
ALL	5.64	41.493
D	9.23	26.16

Figure 7. Effect of ONX 0914 on SIINFEKL expression. RBC-lysed splenocytes from C57BL/6-Tg(CAG-OVA)916Jen/J mice were treated with ONX 0914 (300 nM) or vehicle (DMSO, .1%) for 1 hour. Cells were incubated (at 37°C and 5% CO_2) for 3 hours, 24 hours, and 48 hours post-treatment and analyzed using flow cytometry to determine the percentage of SIINFEKL expression. To minimize readout skewing due to cell death, cells were subjected to gradient separation with lympholyte M to obtain purified viable cells. (A) The percentage decrease was calculated as ([%SIINFEKL in DMSO-%SIINFEKL in ONX 0914 treated splenocytes]/[%SIINFEKL in DMSO]). Experiments were repeated twice, with 2 to 3 spleens per condition. Statistical analysis was performed using 1-way ANOVA to compare percentage decrease in SIINFEKL expression at different time points followed by Tukey's multiple comparison test on individual pairs when ANOVA test was statistically significant ($P < .05$). By 3 hours the decrease in SIINFEKL expression was 16.31% ± 2.4%, at 24 hours it peaked at 26.94% ± 4.10%, and by 48 hours by 23.47% ± 5.03%. *$P < .05$ (between 3 and 24 hours). (B) Representative histogram plot on the effect of ONX 0914 on SIINFEKL expression. At 24 hours the percentage decrease in SIINFEKL expression was 25.5% (from 74.5% in DMSO versus 55.5% in ONX 0914). (C) The mean fluorescence intensity (MFI) was also considerably decreased after ONX 0914 treatment. In region D the decrease was of 26.16%, whereas the entire MFI shifted 41.93%.

MLC where responder, stimulator, or responder and stimulator cells were treated before culture. Also, we varied the treatment sequence so that irradiation of stimulator cells was performed either before or after treatment to emulate the in vivo condition wherein administration was performed post-irradiation (ie, first dose was given post-irradiation and after ATBM and lymphocyte transplantation. Then, we postponed the use of treated stimulators for 24 hours to evaluate if maximal effects could be achieved by pretreatment before culture. The results from these experiments indicated that ONX 0914 preferentially modulated stimulator cells, whereas treatment of responder cells before MLC had no effect on IFN-γ production (Figure 2B); and that a 24-hour lapse between the exposure of stimulator cells to ONX 0914 before initiation of the MLC did not completely abrogate the production of IFN-γ by allogeneic B10.BR T cells against treated B6 splenocytes. However, the data suggested that it provided more suppression (~77% versus 60% when cells were plated immediately after treatment).

To assert the specificity of LMP7 immunoproteasome inhibition, we compared the effects of ONX 0914 with PR-825 (an inhibitor of the constitutive proteasome). The results indicated that allogeneic responses in both MHC-mismatched and miHA-disparate models tested could be suppressed upon ONX 0914 treatment of stimulator cells but remained unaffected in PR-825 treated cultures (Figures 3 and 4). Furthermore, because both GVHD and GVL responses in the B10.BR→CBA BMT model are mediated by

$CD8^+$ T cells [22], we studied specifically the response of $CD8^+$ B10.BR T cells in MLCs against ONX 0914 or PR-825–treated CBA splenocytes and confirmed that only LMP7-specific inhibition led to a decreased production of IFN-γ by this population (Figure 4).

ONX 0914 has been shown to block cytokine production by activated lymphocytes [6,7,23]. We thus investigated whether ONX 0914 treatment would result in a consequent reduction of IFN-γ production by allo-stimulated responder T cells but found no significant difference. At the same time, we corroborated that ONX 0914–treated CD3/CD28 stimulated T cells produced significantly less IFN-γ (~50% less), similar to that reported in the literature [7] (Figure 5). As a possible explanation to the observed discrepancy between allogeneic and polyclonal responses, we hypothesize that either allogeneic and polyclonal stimulation work differentially on immunoproteasome activity of T cells or that the effect of a 1-hour exposure to ONX 0914 on alloreactive T cells was lost or rendered ineffective because of the much slower activation process associated with normal antigen recognition (which may take 6 to 18 hours to occur). Although this latter scenario would have also likely affected the MLC in which stimulator cells alone were treated, we speculate that the exposure of splenocytes to high-dose irradiation (15 Gy) may have hindered the reinstitution of the inhibited immunoproteasome activity, accounting for the observed results. Similarly, in vivo administration of ONX 0914 may have had a minimal effect on allo-antigen stimulated T cells. On the other hand, effects on recipient antigen-presenting cells were more impactful although limited by the proteosomal reconstitution of radio-resistant host cells capable of reinstating LMP7 activity after cessation of treatment.

Finally, because the immunoproteasome can shape both endogenous and virally derived MHC class I–restricted antigen presentation [7,20,24], we investigated the effects of ONX 0914 on the endogenously expressed SIINFEKL peptide. Although to the best of our knowledge there has been no study demonstrating the LMP7-dependency of the OVA257–264 epitope, we decided to use splenocytes from transgenic B6-SIINFEKL mice because this peptide is known to generate $CD8^+$ T responses—the population largely responsible for GVHD in the B10.BR→ CBA model [22]. Interestingly, we found that the percentage decrease of SIINFEKL expression peaked at 24 hours after a 1-hour exposure to ONX 0914 and that this decrease was less prominent by 48 hours (Figure 7).

In addition to down-regulation of IFN-γ production, ONX 0914 can inhibit IL-23 and TNF-α production, among other cytokines [6,7,23], in an NK-κB–independent manner. Reduction of TNF-α in particular was shown to be associated with bortezomib-induced amelioration of GVHD [9], suggesting that reduction of cytokines associated with the development of this disease is a plausible mechanism for the improved survival of CBA recipients in our model. Of note, a pilot proliferation study (Supplemental Figure 1) also showed that donor T cells displayed a lower proliferative tendency when recipient mice were treated with ONX 0914 or bortezomib. Particularly, ONX 0914 had a greater percentage of $eFluor670^{high}$ cells. Nevertheless, bortezomib had by far a lower ratio of $eFluor670^{low}/(eFluor670^{low} + eFluor670^{high})$ cells in both the $CD4^+$ and $CD8^+$ populations but particularly in the CD8 group, suggesting, as previously described, that bortezomib induces cell apoptosis of alloreactive and dividing T cells [9]. Likewise, we also noted that ONX 0914 exposure (ie, 1 hour in vitro treatment as described) did not induce cell death (data not shown), as previously reported to be the case after in vivo administration in naive mice [7]. Furthermore, we did not see a reduction of IFN-γ–producing spot counts when T cells alone were treated before MLC; therefore, we believe the ONX 0914 treatments (both in vitro and in vivo) did not induce significant apoptosis of alloreactive T cells.

Together with the fact that proteasome-mediated proteolysis is responsible for the generation of immunogenic epitopes presented by MHC class I molecules, necessary to activate antigen-specific $CD8^+$ T cells [24], our data support that LMP7 inhibition in the context of BMT modulates allogeneic responses by decreasing endogenous miHA presentation and that the consequential reduction in allostimulation and cytokine production reduces GVHD development. Finally, in lieu of its potential effects on antigen presentation, immunoproteasome inhibition will also need to be tested in tumor-bearing mice to ensure the benefits presented here for GVHD are not detrimental to the GVL potential of the transplant.

ACKNOWLEDGMENTS

Financial disclosure: Funding for this study was provided by Onyx Pharmaceuticals Inc., an Amgen subsidiary.

Conflicts of interest statement: The authors have no conflict of interest to declare.

Authorship statement: J.Z. and J.M. contributed equally to this work.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2015.06.010.

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